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DETERMINATION OF CHIRALITY OF ALCOHOL OR LATENT ALCOHOL SEMIOCHEMICALS IN INDIVIDUAL INSECTS

K.N. SLESSOR,[1] G.G.S. KING,[1] D.R. MILLER,[2] M.L. WINSTON,[2] and T.L. CUTFORTH[1]

[1]*Department of Chemistry*
[2]*Centre for Pest Management, Department of Biological Sciences, Simon Fraser University, Burnaby, B.C., Canada V5A 1S6*

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Abstract—A method is described for determining the enantiomeric composition of chiral alcohols, lactones, and hydroxy acids in quantities ranging from 25 ng to 10 μg. Derivatization of the substance with chirally pure acetyl lactate, followed by splitless capillary gas chromatography, enables enantiomeric determinations to be made within 1-3% of the actual value. This technique was applied in the determination of semiochemical in *Ips pini* (Say), *Apis mellifera* (L.), and *Cryptolestes ferrugineus* (Stephens). The results indicate that considerable variability exists within populations of some insects in the composition of their chiral semiochemicals, whereas others produce substances of constant composition.

Key Words—Chiral semiochemicals, pheromones, enantiomeric composition, *Ips pini*, Coleoptera, Scolytidae, *Apis mellifera*, Hymenoptera, apidae, *Cryptolestes Ferrugineus*, Cucujidae, acetyl lactate diastereoisomers.

INTRODUCTION

Many insects utilize the chirality inherent in some semiochemicals as one of a number of means to encode their messages. Determination of chirality has, however, been limited to the sampling of large numbers of insects due to the small amounts of semiochemical present in individual insects. Typically, a large number of insects would be gathered, the semiochemical extracted and purified in a single sample, and the determination of chirality made by either derivative formation, followed by chromatographic (Fish et al., 1984), spectroscopic anal-

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ysis (Plummer et al., 1976), or by optical rotation measurements (Silverstein et al., 1966). If analyses indicated enantiomeric mixtures, very little could be inferred regarding the production of semiochemical within individuals and a question remained as to whether the individuals in that population were uniform or variable in the chirality of the semiochemical they produced.

Splitless capillary gas chromatography (SCGC) permits measurement of small amounts of pheromone present in individual insects due to the high sensitivity and excellent resolving capability of this technique. The direct separation of enantiomers (Schurig et al., 1983) has been achieved but requires the use of chiral capillary columns that are either very expensive or unavailable. Formation of diastereomeric derivatives using chirally pure acetyl lactate (see, for example, Gil-Av and Nurok, 1974) and separation of diastereomers by conventional splitless capillary gas chromatography (Doolittle and Heath, 1984) offers an attractive alternative. We report modifications of this technique that utilize purified reagents and solvents to convert chiral alcohols and their derivatives into separable diastereoisomers, enabling chiral determinations to be made on unpurified extracts of individual insects.

METHODS AND MATERIALS

Synthetic Semiochemicals. The solvents used were analytical reagent grade and were freshly distilled before use. Chiral 2-nonanols were prepared by CuI-catalyzed hexyl Grignard ring opening of chiral methyloxiranes as described for chiral sulcatol, 6-methyl-5-hepten-2-ol (Johnston and Slessor, 1979), and chiral 9-hydroxy-(*E*)-2-decenoic acids were synthesized using Kandil and Slessor's (1983) method. Racemic ipsdienol, 2-methyl-6-methylene-2,7-octadien-4-ol, was obtained from Borregaard, A.S., Sarpsborg, Norway. Chiral ipsdienol and 11-*S*-(+)-(*Z*)-3-dodecen-11-olide were kindly supplied by E.K. Czyzewska, J.G. Millar, and A.C. Oehlschlager, Department of Chemistry, Simon Fraser University.

Insect Extracts. Individual male pine engravers, *Ips pini* (Say), from a laboratory colony established with beetles from the east Kootenay region of British Columbia, were placed on bolts of lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, and restrained by gelatin pill capsules (Borden, 1967). After a feeding period of 24–48 hr, beetles were removed from the phloem and their abdomens were excised. Ipsdienol was extracted by placing an individual abdomen in a pentane solution (100 μl) of racemic 3-octanol (internal standard, 4.1 ng/μl) in a 1.9-ml shell vial and macerating it with a microspatula for 15 sec. The extract, as well as a wash (50 μl) with the same 3-octanol solution, was transferred to a 1.9-ml screw-cap vial and placed immediately on Dry Ice. Samples were stored at $-70°C$.

To obtain 2-nonanol, which has been reported as an alarm substance in the

honeybee, *Apis mellifera ligustica* (L.) (Collins and Blum, 1982), individual foraging workers were chilled to −10°C for 0.5 hr. The ventral side of the abdomen was stroked to expose the sting. The sting and its associated gland were removed with forceps and extracted immediately with an ether solution (10 μl) of racemic 3-decanol (internal standard, 50 ng/μl). The individual extracts were stored on Dry Ice.

9-Hydroxy-(*E*)-2-decenoic acid, a queen-produced honeybee attractant (reviewed by Winston et al., 1982), was extracted from the mandibular glands of mated, individual honeybee queens, *A. mellifera ligustica*, by dissecting and placing the glands in an ether solution (20 μl) containing racemic 3-decanol (internal standard, 50 ng/μl). The glands were gently macerated with a microspatula and the supernatant esterified immediately with boron trifluoride–methanol.

Individual rusty grain beetles, *Cryptolestes ferrugineus* (Stephens), retain insufficient amounts of their lactone pheromones to analyze (Wong et al., 1983). Therefore, pentane extracts of Porapak Q-collected volatiles from feeding adults were obtained from H.D. Pierce, Jr., A.M. Pierce, and A.C. Oehlschlager, Department of Chemistry, Simon Fraser University.

Acetyl S-Lactyl Chloride Reagent. *S*-(+)-Lactic acid, L-1750, (Sigma Chemical Company, St. Louis, Missouri) (2.7 g, 30 mmol) was dissolved in freshly distilled acetyl chloride (5 g, 64 mmol) and the solution kept at room temperature for 2 hr. Excess acetyl chloride was evaporated in vacuo, and thionyl chloride (10 g, 84 mmol) was added. The solution was left overnight at room temperature and evaporated to yield crude acetyl lactyl chloride (3.6 g) containing approximately 10% dimeric and trimeric species. Pure monomer (1.2 g) was obtained by careful bulb-to-bulb vacuum distillation on a water aspirator (12 mm, 50–60°C bath temperature). The reagent solution was prepared by dissolving the distillate (25 mg) in dry methylene chloride (1 ml). The reagent solution was stored at 2°C in Teflon-lined screw-cap glass vials and was stable for several months.

Derivatization of Chiral Alcohols. Solutions of 2-nonanol in hexane were prepared in various enantiomeric ratios and used as standards. Extracts of *I. pini* containing >25 ng of ipsdienol were concentrated to approximately 20 μl by evaporation at room temperature. Extracts of honeybee sting glands containing >150 ng of 2-nonanol were derivatized directly. To a solution of the standard alcohol (1–10 μg) or an insect extract (10–30 μl) placed in an ampoule prepared from a fresh disposable pipet was added a 50 mg/ml pyridine in ether solution (15 μl) followed by the acetyl *S*-lactyl chloride reagent (30 μl). The components were mixed thoroughly, and the solution was cooled to −20°C and carefully sealed. Ampoules were kept at room temperature overnight, then opened and diluted with hexane (50 μl). The solution was washed by adding water (50 μl), agitated to ensure mixing, and allowed to settle. The aqueous phase was removed, and the organic phase was further washed with aqueous 5%

sodium bicarbonate (3 × 50 μl) and finally once more with water (50 μl). The sample was diluted with hexane to an appropriate concentration for analysis by SCGC. For smaller amounts of alcohol (25–1000 ng), the quantities of the reagents were reduced by one third.

Derivatization of Hydroxy Acids and Lactones. Standards, queen mandibular extracts, and rusty grain beetle isolates (0.5–5 μg) were dissolved in methanol (50–100 μl) containing 5% boron trifluoride–etherate, cooled to −20°C, and sealed in glass ampoules. Hydroxy acids were kept overnight at room temperature, whereas lactones were heated at 70°C overnight to convert to the hydroxy ester. Work-up involved dilution with pentane (100 μl) and washing with water (2 × 150 μl, 2 × 50 μl). An aliquot was taken for SCGC analysis, and the remainder was dried over finely divided anhydrous sodium sulfate, concentrated in an air stream, and derivatized as described for alcohols.

Analyses. Splitless capillary gas chromatography (SCGC), was carried out on a Hewlett Packard HP 5890 using 30-m × 0.25-mm ID fused silica column, with injector and detector temperatures of 250°C. The column, a methylsilicone DB-1 (J + W Scientific, Inc., Rancho Cordova, California), was temperature programmed as indicated in Table 4. Flame ionization detection was employed with a helium carrier and makeup gas. Kinetic resolution was investigated using several separate derivatizations of racemic 2-nonanol in hexane and working up the samples at intervals.

Analyses of standards and representative and unusual insect derivatives were routinely run on a Hewlett Packard HP 5985B splitless capillary gas chromatograph–mass spectrometer. The mass spectra of the separated diastereomeric derivatives were compared and always found to be nearly identical, disclosing no underlying impurities.

RESULTS AND DISCUSSION

Analysis of a variety of racemic alcohols by the SCGC acetyl lactate method generally resulted in the baseline separation of the two diastereoisomers with nearly equal intensities, as measured by flame ionization detection. Chirality determination of derivatized 2-nonanol and sulcatol over a range of enantiomeric mixtures were in good agreement with the chirality of the prepared mixtures (Table 1). When chiral mixtures of defined composition were analyzed in five separate experiments, the method proved to be highly reproducible (Table 2). The 3-octanol utilized as an internal standard in the chiral determinations of ipsdienol from *I. pini* was shown to be 51.05% *R*-(−) (SE = 0.15%) for 132 determinations. The presence of only one peak when a pure enantiomer was derivatized indicates the high purity of the lactate reagent as well as the alcohol. Each new preparation of the lactate reagent was checked with a pure chiral alcohol to ensure that partial racemization of the reagent had not occurred during preparation.

TABLE 1. DETERMINATION OF PERCENT *S*-(+)-2-NONANOL AND *S*-(+)-6-METHYL-5-HEPTEN-2-OL IN ENANTIOMERIC MIXTURES BY SCGC OF ACETYL *S*-(+)-LACTYL DIASTEREOISOMERS

S-(+)-enantiomer prepared (%)	100 ng sample, *S*-(+) enantiomer determined (%)	900 ng sample, *S*-(+) enantiomer determined (%)
2-Nonanol		
0	0.8	0.0
15	15.0	16.5
30	29.3	29.9
50	49.0	48.8
70	69.4	70.1
85	83.9	83.4
100	99.3	99.0
6-Methyl-5-hepten-2-ol		
0	0.9	0.6
15	15.1	14.0
30	30.1	29.0
50	48.7	46.9
70	68.8	66.4
85	84.6	82.5
100	100.0	99.4

TABLE 2. REPRODUCIBILITY OF CHIRAL DETERMINATION ON TWO SOLUTIONS OF 2-NONANOL: SOLUTION 1, 75% *S*; AND SOLUTION 2, 82% *S*[a]

Trial	Solution 1, *S*-2-nonanol determined (%)	Solution 2, *S*-2-nonanol determined (%)
1	74.5	84.4
2	74.3	81.3
3	74.1	82.8
4	73.8	82.1
5	74.7	81.6
Mean ± SE	74.3 ± 0.2	82.4 ± 0.6

[a]Each trial analyzed 0.5-μg samples of the test solutions.

For the simple acyclic alcohols used in this study, there was only minimal kinetic resolution found when derivative formation was incomplete (Table 3). Thus, in the inadvertent case of incomplete reaction, the derivative ratio would deviate less then 5% from the original enantiomeric ratio.

Of the 54 male *I. pini* producing more than 25 ng of ipsdienol, the enantiomeric composition averaged 94% *R*-(−)-ipsdienol, in complete agreement

TABLE 3. KINETIC RESOLUTION OF RACEMIC 2-NONANOL IN INCOMPLETE DERIVATIZATIONS[a]

Completion (%)	*S*-(+) enantiomer determined (%)
25	47.0
34	47.8
40	47.6
70	48.1
89	48.9
100	49.9

[a]Reaction begun with 0.5 μg of racemic 2-nonanol and % completion analyzed by SCGC on program a. See footnote *a* Table 4.

with pooled estimates for California (Birch et al., 1980) and Idaho populations (Plummer et al., 1976). However, considerable individual variation occurred in this population (Figure 1). Geographic variation in the production of and response to chiral ipsdienol has been clearly demonstrated for populations of *I. pini* obtained from separate locales (Birch et al., 1980; Lanier et al., 1980). Individual variation in chirality of pheromones, as shown by *I. pini* from a single population, may have profound implications both for population studies and for pest management programs. New populations of insects could arise in response to natural or artificial selection pressures, such as natural catastrophies or pheromone-based mass trapping programs.

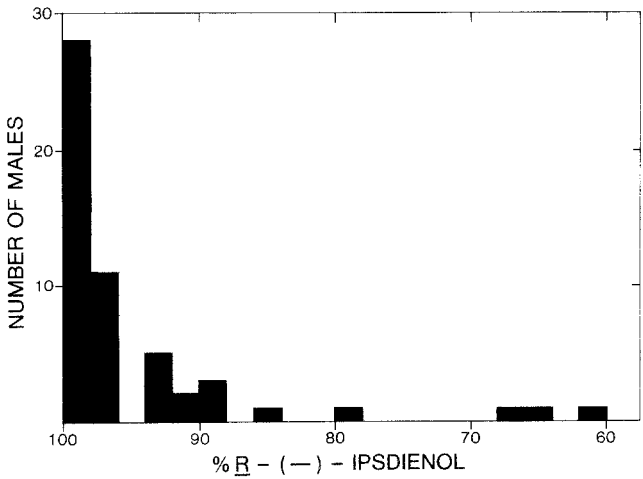

FIG. 1. Chirality of ipsdienol produced by individual male *Ips pini* from the east Kootenay region of British Columbia.

TABLE 4. RETENTION TIMES AND MASS SPECTRAL CHARACTERISTICS OF ACETYL *S*-(+)-LACTYL DIASTEREOMERIC DERIVATIVES

Compound	Program[a]		Free alcohol time (min)	Acetyl *S*-lactate time (min)	Predominant mass spectral fragments of *S*-(+)-lactates *m/e* (% abundance)
R-(−)-2-Nonanol	a		7.86	16.86	115(100), 43(91), 71(86),
S-(+)-2-Nonanol				17.08	88(85), 87(83), 85(60), 133(35), 126(15)
R-(−)-Sulcatol	b		5.26	15.30	95(100), 110(80), 69(47),
S-(+)-Sulcatol				15.62	43(39), 87(15), 115(10)
S-(+)-Ipsdienol	c		5.92	19.30	87(100), 43(97), 119(89),
R-(−)-Ipsdienol				19.61	115(65), 134(65), 91(55), 85(55)
Methyl *R*-(−)-9-hydroxy-(*E*)-2-decenoate	d		19.29	37.88	81(100), 115(95), 87(84), 123(82), 113(60), 150(50),
Methyl *S*-(+)-9-hydroxy-(*E*)-2-decenoate				38.64	133(20), 184(15)
11-*R*-(−)-(*Z*)-3-Dodecen-11-olide	e	Lactone	15.16	29.99	81(100), 87(95), 67(85), 55(78), 115(65), 84(62),
11-*S*-(+)-(*Z*)-3-Dodecen-11-olide		Methyl ester	19.50	30.42	95(55), 136(55), 137(45), 210(12)

[a] SCGC was performed on a 30-m × 0.25-mm ID fused silica methylsilicone (DB-1) capillary column programmed as follows: a, 60°C for 2 min, 7°C/min to 240°C; b, 60°C for 2 min, 7°C/min to 120°C, 2°C/min to 210°C; c, 80°C for 2 min, 10°C/min to 120°C, isothermal for 5 min, 2°C/min to 240°C; d, 60°C for 2 min, 7°C/min to 130°C, 2°C/min to 240°C; and e, 60°C for 2 min, 7°C/min to 180°C, 2°C/min to 210°C.

The chirality of the 9-hydroxy-(*E*)-2-decenoic acid in 22 individual honeybee queens varied from 70 to 95% *R*-(−), with a mean of 84.8 ± 1.8%. These results are in accord with experiments examining behavior elicited by 9-hydroxy-(*E*)-2-decenoic acid, which showed the *R*-(−) enantiomer to be more effective than its antipode in the settling response of queenless honeybee swarms (Winston et al., 1982). Worker honeybees from two hives yielded 2-nonanol with a very high predominance (>95%) of the *S*-(+) enantiomer. The complexity of the semiochemical arsenal of the worker honeybee sting gland (Blum et al., 1978) prevented a more accurate analysis due to overlapping peaks of minor components when analyzed on either column.

The isolates obtained from rusty grain beetle volatiles contained 11-*S*-(*Z*)-3-dodecen-11-olide in greater than 99% enantiomeric purity. This result is entirely consistent with the finding of Wong et al., (1983), that the insects' aggregation response is initiated by the 11-*S* enantiomer.

Our results demonstrate that the chirality of alcohol and latent alcohol semiochemicals can be accurately determined for individual insects. Retention times and characteristic mass spectral fragments of these semiochemical derivatives are reported in Table 4. Determination of the chirality of semiochemical in individual insects will provide a means of examining the variability and monitoring changes in the production of chiral semiochemicals.

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